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Rapid Onset of the Accelerated Degradation of Dicarboximide Fungicides in a UK Soil with a Long History of Agrochemical Exclusion

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Abstract: In field and laboratory experiments, enhanced degradation of the dicarboximide fungicides, iprodione and vinclozolin, was stimulated by only one application of the fungicides in a soil with no previous history of any pesticide input. Field and laboratory studies demonstrated the ease of stimulation by pre-treatment with even very low concentrations of the fungicides (0·5 $\mu g\ g^{-1}$ soil) and at a range of temperatures and soil moisture conditions. Soils that had acquired full enhanced degradation could rapidly degrade fungicide applied at 30 times the recommended field rate. Cross-enhancement of degradation was noted with both fungicides, but not with their common metabolite, 3,5-dichloroaniline. Application of the antibiotics chloramphenicol or rifampicin to soil reduced enhanced degradation to control levels; cycloheximide had no effect. This, together with the inhibitory action of azide, mercuric chloride and repetitive microwaving, indicated that the agent(s) of enhanced degradation was probably bacterial.

Key words: accelerated degradation, dicarboximide fungicides, iprodione, vinclozolin, bacterial agents, cross-enhancement, inhibition by antibiotics, environmental factors

1 INTRODUCTION

Enhanced (accelerated) degradation has been defined as the increased rate of decomposition of a pesticide in soil, induced by a previous treatment regime with the same pesticide.[1–3] In the UK, accelerated degradation has been associated with the failure in field performance of several insecticides, nematicides and fungicides following their repeated application to the same soils.[4–9]

The enhanced degradation of the dicarboximide fungicides, iprodione and vinclozolin, was first demonstrated over ten years ago in several cultivated soils, mainly in central and southern England, where the fungicides had been applied to the soil to control onion white rot.[4,10–12] It has now been observed in a New Zealand soil.[13] Once established, such enhancement is known to persist for years.[9] As part of a collaboration with Horticulture Research International, Wellesbourne, UK, ultimately aimed at the in-situ measurement by molecular techniques of changes in populations of xenobiotic-degrading soil micro-organisms following soil treatment with xenobiotics, we undertook the examination of factors contributing to the appearance of enhanced degradation of these fungicides in a cultivated soil which had a long history of no pesticide, herbicide or fungicide treatments of any kind and the pH of which would result in minimal chemical (abiotic) hydrolysis of the dicarboximide structure.[11] Such a soil would be expected to contain a negligible population of micro-organisms adapted to these fungicides, against which changes following a fungicide amendment could be measured.

2 MATERIALS AND METHODS

Analytical and technical grade samples of iprodione [3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] were provided by Rhône-Poulenc Agrochemie, Lyon, France. Analytical and technical

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grade vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione] were provided by BASF Ltd, Ipswich, Suffolk, UK. Formulated iprodione ('Rovral') and vinclozolin ('Ronilan'), as used agriculturally, were commercial wettable powders, containing approximately 500 g kg^{-1} of the active ingredients; they were supplied by Mr D. L. Suett and Dr A. Walker of Horticulture Research International, Wellesbourne, Warwickshire, UK. Analytical grade 3,5-dichloroaniline (3,5-DCA) was purchased from Aldrich Chemicals UK.

2.1 Field studies

Two plots were prepared; the first was an area within the walled garden (5-m high walls) at Newcastle University's field station; this site has a history of no pesticide, herbicide or fungicide treatment (see Section 2.2). The second site was a horticultural plot outside the walled garden where agrochemicals have been used routinely during this period, although there is no record of the application of any dicarboximide fungicides. Both sites were cleared of vegetation, rotovated and then dug to full spade depth to break up the sub-surface pan. Rows of 5 m length, 1 m apart, were treated with one of the two commercial fungicide wettable powders, 'Rovral' or 'Ronilan', at 0·3 g active ingredient (AI) suspended in 1 litre of water, for each metre of row; alternate rows were left untreated (water only applied). Application by watering can with a small nozzle gave a drench application width of about 5 cm along the rows. Rows on half of the sites were sown with salad onion (*Allium cepivorum* cv. White Lisbon) to simulate normal horticultural practice; the remaining rows were left crop-free. The onion crop was harvested after 11 weeks and a second crop immediately sown along the same crop rows. Thus, in the alternate rows where fungicide was again applied, there were two treatments of fungicide on that row during the growing season. Surface soil samples from fungicide-treated and untreated rows were taken to a maximum depth of 10 cm, with separate small trowels for each type of treatment, the trowel cleaned by rinsing in tap water, acetone which was flamed off and then distilled water, between successive samples. The samples were well mixed by tumbling in a ball mill for 3 min and replicate portions analysed for fungicides and their metabolites.

2.2 Soil incubation studies

Soil used in the laboratory studies, obtained from the first field site (Section 2.1), was of the Brown Sand (Newport Series) type but modified by at least 200 years of cultivation within the walled garden of the Close House estate, since 1961 the experimental field station of Newcastle University at Heddon-on-the-Wall, Northumberland, UK. There has been no treatment of this soil or its crops with any pesticide, herbicide or fungicide for at least 35 years although it is cultivated and cropped regularly. Although drift of air-borne agrochemicals from arable farms in the locality cannot be entirely excluded, it is probably negligible because the experimental site is immediately surrounded by several hundred acres of woodland and amenity grassland which do not receive agrochemicals. The soil from within the agrochemical-free walled garden plot (Section 2.1) had a mechanical composition ascertained by the pipette/sedimentation procedure of 20·6% clay, 14·8% silt and 64·6% sand. It contained 4·8% (w/w) total organic carbon, had a pH (measured in water) of 6·3 and a water-holding capacity (WHC) of 32% (w/w). A sample of the fresh soil was dried at 110°C in an oven to constant weight to determine its moisture content and enable adjustments of moisture content to be made for other experiments (Section 2.3.2). Available nitrogen (extracted in potassium sulfate) was 33·2 mg kg^{-1} and total nitrogen (Kjeldahl) 3·49 g kg^{-1}; available potassium (ammonium nitrate method) was 100 mg kg^{-1} and available phosphorus (Olsen method) 325 mg kg^{-1}. These N, P and K values[14] were not supplemented by mineral or organic fertilisers during the course of the study. Fresh soil, taken from the top 10 cm of the walled garden site, was passed through a sieve with 2-mm diameter mesh and the sieved soil pretreated in one of three ways before use in laboratory experiments:

(i) One sample was covered with 'cling film' and exposed to microwaves (three 1-min exposures at 600 W with 5-min intervals), cooled, incubated at 23°C for five days and re-exposed to microwaves with a further five-day incubation at 23°C before the third and last microwave treatment. The procedure was completed just prior to an experimental study to produce a soil which was kept covered until it was used within 6 h.

(ii) Other soil batches were mixed with an aqueous suspension of formulated fungicide (vinclozolin or iprodione) to give a final concentration of 5 μg AI g^{-1} dry weight of soil and adjusted to 20% (w/w) moisture content (Section 2.3.2). They were then incubated for 14 days at 23°C. This fungicide treatment was repeated three times at two-week intervals and a soil sample analysed at the end of the last treatment to check that all fungicide from the previous treatments had disappeared before the soil was used in subsequent experiments. If residues were present, incubation was continued until they disappeared or the soil sample was discarded.

(iii) A fourth batch of fresh soil was amended with water instead of the aqueous suspension of fungicides, but otherwise treated the same as the fungicide-treated samples. These procedures thus gave four soil types: microwave-treated, vinclozolin-pretreated, iprodione-pretreated and untreated.

Experimental soil incubations were carried out in glass dishes [5 cm deep × 9·5 cm diam., holding soil

samples (100 g)], six of which were stacked one above the other in closed glass containers.[15] The dishes of soil in the glass containers were pre-incubated for 24 h at the appropriate temperature. Aqueous suspensions of a formulated fungicide (at the test concentration of 25 μg AI g^{-1} dry weight of soil) and any other additions were then added to the soil and mixed mechanically. The efficiency of the mixing procedure was determined by taking six samples from different parts of a test dish and analysing the fungicide concentration by HPLC (see below). Variations within $\pm 5\%$ of the mean were regarded as acceptable. After initial adjustment, the dishes of soil were routinely maintained at a constant moisture content of 20% (w/w) by readjusting the dishes at two-day intervals to their initial moisture weight with distilled water. Triplicate samples (10 g) of soil were removed from dishes at intervals for fungicide extraction and HPLC analyses.

2.3 Factors affecting fungicide degradation in soil

For investigations of some of the parameters affecting enhanced degradation of the fungicides, the standard procedures were modified as follows:

2.3.1 Temperature effects

Fungicide degradation was examined at incubator temperatures of 23, 12 and 4°C. To achieve a soil temperature of 0°C, the soil jars were immersed in crushed ice in a pre-cooled insulated container held in a refrigerator. This soil temperature was regularly checked by suspending a mercury thermometer bulb in the soil of one dish in the container which was read daily during the sampling regime.

2.3.2 Soil moisture content

A range of soil moisture levels [5–30% (w/w)] was attained by adjusting the moisture content of fresh soil (determined from its dry weight measurement, Section 2.2) and maintained by daily addition of water to the original fresh weight of the individually modified soils. Dry soil was produced by drying fresh soil for seven days in a 30°C incubator then standing it over phosphorus pentoxide in a desiccator at 23°C to constant weight. Subsequent experiments with this dry soil were incubated in the same desiccator over phosphorus pentoxide. After the time course of this experiment was complete, some of the dry soil dishes (approx. 0% free moisture content) were removed to the laboratory and remoistened to 20% (w/w) water content, after which sampling and fungicide analyses were continued for a further three days.

2.3.3 Effect of introducing pretreated soil into untreated soil

Pretreated Close House soils at 20% (w/w) moisture content, actively degrading iprodione or vinclozolin, respectively, were mixed with untreated soil at the same moisture content to give experimental soil mixtures containing between none and 100% (w/w) of the appropriate pretreated, fungicide-degrading soil. These soil mixtures were incubated for 20 days at 23°C in jars, with their moisture content maintained at 20% (w/w) and then checked for absence of any fungicide residues from the pretreatment protocols. The appropriate experimental fungicide was added at the standard test concentration (25 μg AI g^{-1} dry weight of soil), mixed, and its residual concentrations in the soil followed for at least two weeks.

2.3.4 Pretreatment with different fungicide application rates

To examine the enhancing effect of pretreatment with low and very high application rates of the fungicides, samples of untreated soil from Close House were incubated with iprodione or vinclozolin at initial concentrations of 0·5 to 700 μg AI g^{-1} dry weight soil. After incubation at 23°C in the dark, until this single treatment amount of fungicide had just disappeared, further fungicide at the standard test concentration (25 μg AI g^{-1} dry weight of soil) was then added to all the dishes and its residues measured during the subsequent 16 days.

2.3.5 Cross-enhancement experiments

Formulated fungicide (25 μg AI g^{-1} dry weight of soil) as aqueous suspensions, or 3,5-DCA (25 μg g^{-1} dry weight of soil) dissolved in acetone (maximum concentration of acetone added to the soil was <5 ml kg^{-1}) was applied to dishes of soil to induce enhanced degradation. To examine possible cross-enhancement, each soil type (untreated, iprodione-pretreated, vinclozolin-pretreated or 3,5-DCA-pretreated) received, three days after any previous residues had disappeared, one of three subsequent amendments: iprodione addition, vinclozolin addition or 3,5-DCA addition (at all 25 μg AI g^{-1} dry weight of soil) giving a total of 12 treatments. The soil dishes were incubated at 23°C in the dark but sampled and analysed for fungicide residues over the next 21 days.

2.3.6 Effect of pretreatment with 3,5-dichloroaniline

To ascertain if pretreatment with, or the presence in the soil of, 3,5-DCA, the common degradation product of the two fungicides, could stimulate degradation of either of the fungicides or of 3,5-DCA itself, 3,5-DCA (in acetone) was added to a final concentration of 25 μg g^{-1} dry weight of untreated soil (maximum volume of acetone added to the soil dishes was $\lesssim 0{\cdot}5$ ml) and incubated for four weeks at 23°C in the dark, after which time iprodione or vinclozolin was incorporated into the soil at the standard test rate of 25 μg g^{-1} dry weight of soil; the soil was then reincubated,

sampled and the fungicide residues analysed for at least 28 days.

2.3.7 Effects of metabolic poisons and antibiotics on fungicide degradation

Pre-treated soils were first amended with iprodione or vinclozolin (25 μg AI g^{-1} dry weight of soil) and the dishes incubated at 23°C for 24 h. Mercuric chloride or sodium azide solutions were added to provide final concentrations of 2 mg g^{-1} dry weight of soil to half the dishes and an equivalent volume of water added to the remainder as controls; dishes were re-incubated and sampled for fungicide residues at regular intervals thereafter.

Because most antibiotics are effective only against actively growing or proliferating micro-organisms, whereas microbial generation times in typical unsupplemented soils are very long,[16–19] aqueous suspensions of antibiotics active against bacteria (rifampicin, streptomycin sulfate, kanamycin, chloramphenicol) or against fungi (cycloheximide or nystatin) were added to soils at 3 mg g^{-1} dry weight of soil, together with yeast extract or glucose (5 mg g^{-1} dry weight of soil) to encourage active microbial growth and thus more effective action of the antibiotics.[20]

Soils were incubated for 24 h at 23°C following this treatment, after which the fungicides were added (at the test amount of 25 μg AI g^{-1} dry weight of soil) to the dishes. The dishes were then incubated and residues of fungicide and 3,5-DCA analysed at intervals.

2.4 Iprodione and vinclozolin extraction and analysis

Soil samples (10 g) from the dishes were extracted by shaking (100 rev min^{-1} in a Gallenkamp orbital shaker) with acetonitrile (10 ml) for 150 min, to extract residual fungicide and metabolites, including 3,5-DCA. Fungicide and 3,5-DCA in the supernatant were determined in a LDC/Milton Roy HPLC system (LDC/Milton Roy, Stone, Staffs, UK) comprising a Constametric 3000 delivery system, a Spectromonitor 3000 variable wavelength UV detector and a CI-10B computing integrator. The system was fitted with a Merck-Hibar Lichrosorb RP-18 column (250 × 4 mm ID; 5 μm particle size) and was equipped with a Gilson model 231 autosampler. The solvent system contained acetonitrile + water, (60 + 40, by volume) delivered at a flow rate of 1 ml min^{-1}. Compounds were detected at 205 nm. Residues for pesticides and their metabolites were quantified using external standards. All compounds investigated gave a linear calibration over the range tested (0–0·3 mM). This method gave recoveries for iprodione of 102(±6)%, for vinclozolin of 94(±6)% and for 3,5-DCA of 92(±4)% and detected 0·2 μg ml^{-1} of these compounds without special concentration techniques.

2.5 Treatment of data

In each experiment where the loss of fungicide was measured, $\log_{10}$ of the residual fungicide amount (μg g^{-1} dry weight of soil) was plotted against incubation time. The gradient of this line was used as a degradation constant ($-k_D$) for fungicide removal. The line of best fit was determined using the GLIM package and the significance of the fit of the data to the linear model was assessed using the D^2 statistic[21] which is analogous to the r^2 used in linear regression. For the $-k_D$ values quoted, D^2 was $> 0{\cdot}900$. Errors between replicate samples within 5% of the means are not shown.

3 RESULTS

3.1 Field experiments

The rate of disappearance of iprodione and vinclozolin in the walled garden soil was always greater in pretreated field rows, where it was coincident with the very early appearance (three days) of the metabolite 3,5-DCA, than in untreated rows, where this metabolite never appeared before 10–12 days (Fig. 1). Following a single treatment with either fungicide, previously untreated field soil showed enhanced degradation of a second and third application, but the rate of fungicide disappearance even of this first application to untreated Close House soil, though initially very slow, accelerated after seven or eight days. In once-pretreated field rows, the two fungicides disappeared completely within 30–35 days during the period May to September. Salad onions sown in the rows at the time of fungicide applications appeared healthy whether in fungicide-treated or

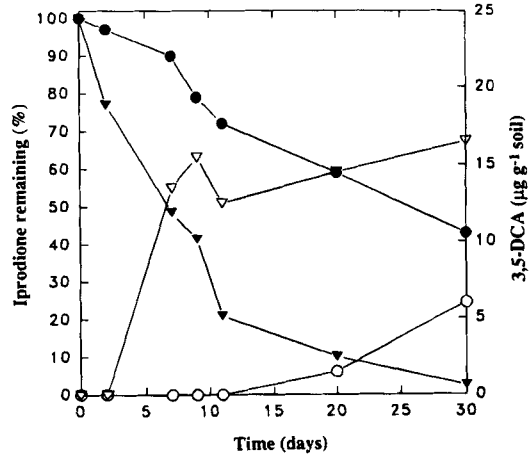


Fig. 1. Degradation of iprodione in field rows, sown with onions, (▼) treated on one previous occasion (six weeks earlier) with iprodione at 0·3 g AI m^{-1} of row (approx. 45 μg g^{-1} of soil), (●) in similar rows never previously exposed to the fungicide. The appearance of the fungicide metabolite 3,5-dichloroaniline was also measured in (▽) pretreated and (○) untreated rows.

untreated rows and their presence had negligible effect on fungicide disappearance, the rate of which was comparable with that in plant-free rows both in the walled garden soil and in the second plot soil (Section 2.1).

3.2 Laboratory experiments on enhanced fungicide degradation

3.2.1 Influence of temperature

In laboratory soil incubations, vinclozolin and iprodione were rapidly transformed by the appropriately pretreated soils at ambient soil temperatures (23°C) with half-lives of <1 to 2 days, respectively ($-k_D$ 0·5–2·0 day^{-1} or greater). Exposing soils to microwaves[22] reduced iprodione and vinclozolin degradation ($-k_D$ 0·008 and 0·01 $days^{-1}$, respectively) in the first 15 days to less than 2% of that seen in an active, fungicide-pretreated soil. Degradation of iprodione eventually redeveloped after 15 days in the iprodione-pretreated, microwaved soil at 23°C at a rate ($-k_D$ 0·30 day^{-1}) approaching that of a normal once-pretreated soil during its period of most rapid fungicide degradation (Fig. 2(a), days 0–4). Iprodione disappearance in untreated Close House soil showed a lag period of slow degradation ($-k_D$ 0·017 day^{-1}) for about six days, followed by a period of faster degradation ($-k_D$ 0·24 day^{-1}) once the degradative ability of the soil was established. A second application of iprodione to this soil gave $-k_D$ 0·52 and a third, $-k_D$ 1·4 day^{-1}. At 12°C, the rate of iprodione degradation in pretreated soil ($-k_D$ 0·30 day^{-1}) was slower than at 23°C ($-k_D$ 0·52 day^{-1}) but the onset of degradation at 12°C in untreated soils and in soils exposed to microwaves was not noticeably retarded compared with that at 23°C, though subsequent rates were slower (Fig. 2(b)). At 4°C, the onset of iprodione degradation in untreated soil was delayed until day 19 or later (data not shown) with a subsequent $-k_D$ value of 0·12 day^{-1}; pretreated soil showed $-k_D$ 0·17 day^{-1}. The $-k_D$ value for iprodione in pretreated soil held at 0°C was 0·035 day^{-1} and the half-life of the iprodione had increased to 28 days (Fig. 2(c)). Untreated soil at 0°C, amended with the fungicides, showed no onset of fungicide degradation within 50 days.

Similar patterns of degradation, with comparable $-k_D$ values, were seen in experiments with vinclozolin in the three types of soil held at these temperatures.

3,5-DCA always accumulated in soils where rapid degradation of the two fungicides had occurred, with the maximum concentration appearing at about the time that 50% of the fungicides had been degraded (Fig. 1), but the stoichiometric conversions of fungicide to 3,5-DCA, seen in liquid cultures of fungicide-degrading bacteria,[23] did not occur in soil. An unidentified metabolite, a precursor of 3,5-DCA also seen in liquid cultures of iprodione-degrading bacteria,[24] appeared transiently in pretreated soil showing rapid degradation of iprodione.

3.2.2 Effect of moisture levels on fungicide degradation rates

Pretreated soil at optimum moisture content (25% (w/w)) displayed rapid fungicide degradation. Even in just water-logged conditions (35% (w/w) moisture content) the loss of fungicide in a pretreated soil at 23°C was almost 90% of the initial application after as little as two days. In soils below 10% (w/w) moisture content, the rates of fungicide degradation were much reduced; in dry (approx 0% moisture content) pretreated soil, iprodione and vinclozolin disappearance was negligible and comparable to that in moist untreated soil (Fig. 4). Remoistening of such dry pretreated soils immediately caused the residual content of either iprodione or vinclozolin to fall. After three days incubation (days 13–15) at 20% (w/w) moisture, following a 12-day dry regime, only 6% of iprodione and 3% of vinclozolin remained of the fungicide concentrations measured in those dry soils at day 12; samples of the same soils left dry showed no further loss of fungicide over this additional three-day period.

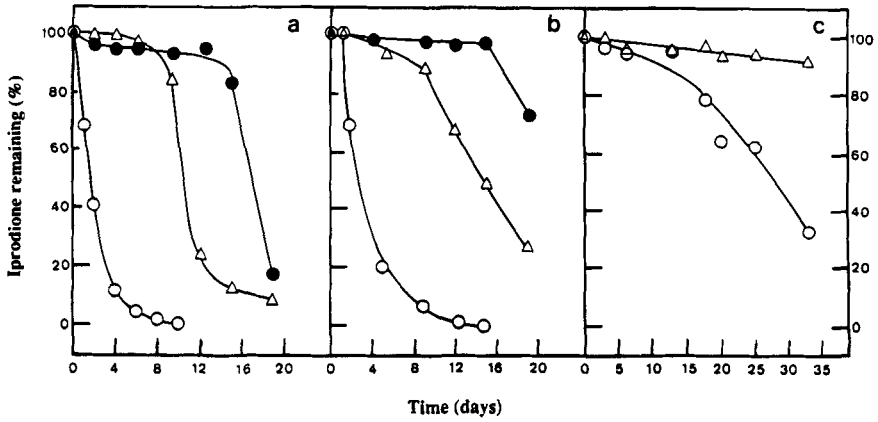


Fig. 2. The effect of temperature on the degradation of iprodione in laboratory incubations of (○) soil previously exposed to the fungicide, (△) untreated soil and (●) untreated soil which had been exposed to microwaves. The experiments were conducted at (a) 23°C; (b) 12°C and (c) 0°C (note change in time-scale).

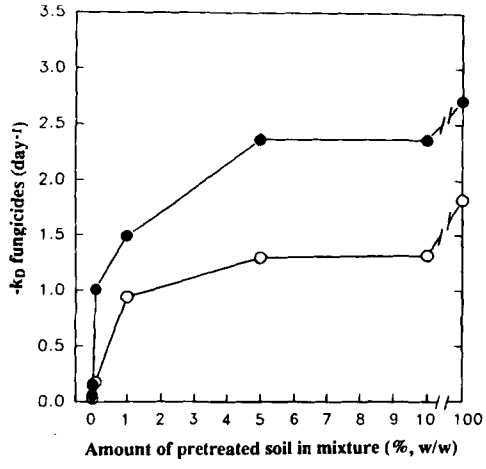


Fig. 3. The effect on the degradation constant ($-k_D$) for (○) iprodione and (●) vinclozolin of mixing and incubating pretreated soil known to be capable of rapidly degrading the respective fungicides, in different amounts with untreated soil never previously exposed to either fungicide. All values of degradation in replicate experiments were within 5% of the mean value.

3.2.3 Mixtures of pretreated and untreated soil

The addition and thorough mixing of as little as 0·1% (w/w) of a fungicide-pretreated soil to an untreated soil consistently and significantly ($P < 0{\cdot}01$) increased the rate of disappearance of the appropriate fungicide compared with the rate in untreated soil alone; this was particularly evident with a vinclozolin-pretreated soil. Additions of more than 5% (w/w) of either iprodione- or vinclozolin-pretreated soils to an untreated soil, produced fungicide degradation rates in the soil mixtures comparable to those obtained in a fully pretreated soil alone (Fig. 3).

3.2.4 Effect of fungicide pre-application rates on subsequent degradation

Pre-incubating untreated soil with increasing concentrations of iprodione or vinclozolin (0·5–10 μg AI g^{-1} of soil) produced a progressive increase in the degradation rate of a subsequent test dose (25 μg AI g^{-1} of soil) of the appropriate fungicide, applied after the pre-incubation dose had disappeared (Fig. 5). When the effect on the soil-degrading potential of pretreatment with much higher concentrations of the fungicides was examined, iprodione-pretreated soil showed rapid degradation of all treatments (100–700 μg g^{-1} of soil) of iprodione (90% loss in two to six days) but with a progressive reduction in rate from the 100 μg g^{-1} of soil treatment ($-k_D$ 2·15 day^{-1}) to the 350 μg g^{-1} of soil treatment ($-k_D$ 0·73 day^{-1}). The highest application of iprodione (700 μg g^{-1} of soil) exhibited a two-day lag before the onset of its maximum degradation rate ($-k_D$ 0·60 day^{-1}). An initial lag in degradation of vinclozolin by vinclozolin-pretreated soil was seen with the two highest applications, but from 100 μg g^{-1} of soil ($-k_D$ 0·58 day^{-1}) to 350 μg g^{-1} of soil ($-k_D$ 0·435 day^{-1}) 90% disappearance of vinclozolin occurred in the subsequent six days; for the 700 μg g^{-1} of soil application of vinclozolin, the degradation rate ($-k_D$ 0·28 day^{-1}) was marginally slower, with 90% of this application removed in nine days.

3.2.5 Cross-enhancement of degradation by the fungicides

The rate of degradation of iprodione in an iprodione-pretreated soil ($-k_D$ 0·79 day^{-1}; 90% loss in two days) was faster than that in vinclozolin-pretreated soil ($-k_D$ 0·39 day^{-1}; 90% loss in four to five days), which in turn was greater than the rate of iprodione degradation ($-k_D$ 0·12 day^{-1}; 90% loss in 15 days) in untreated soil (Fig. 6(a)). Similarly, the degradation of vinclozolin in vinclozolin-pretreated soil ($-k_D$ 0·81 day^{-1}; 90% loss in two days) occurred more rapidly than in iprodione-pretreated soil ($-k_D$ 0·29 day^{-1}; 90% loss required 7·5 days) or in untreated soil ($-k_D$ 0·20 day^{-1}; 90% loss in

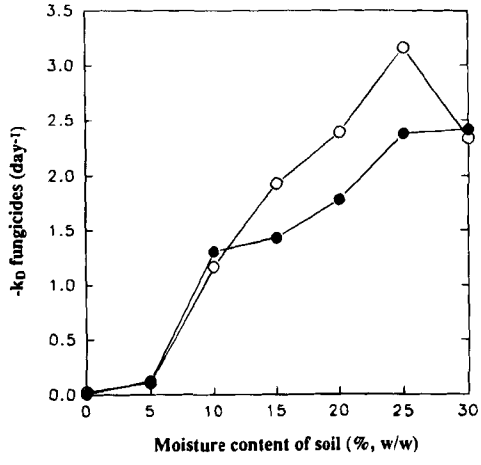


Fig. 4. The effect of soil moisture on the degradation constants ($-k_D$) for (○) iprodione and (●) vinclozolin in fungicide-pretreated soils at 23°C in laboratory incubations.

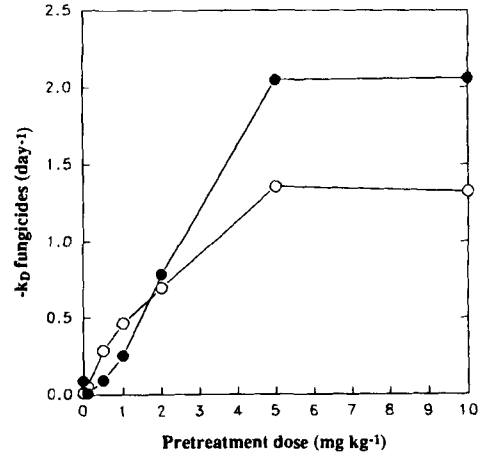


Fig. 5. The effect on subsequent (○) iprodione and (●) vinclozolin degradation constants ($-k_D$) of previous exposure of soils to low concentrations of the same fungicides, respectively (i.e. below the manufacturer's recommended field application rate).

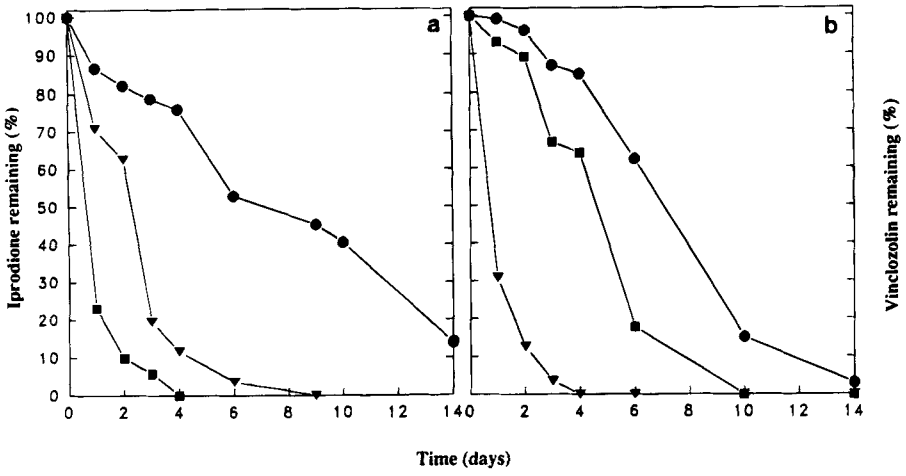


Fig. 6. Evidence of cross-enhancement by the dicarboximide fungicides in laboratory incubations of Close House soil. Iprodione degradation (a) and vinclozolin degradation (b) in (●) soil not previously exposed to any fungicide; (■) soil previously exposed to iprodione and (▼) soil previously exposed to vinclozolin.

12 days) (Fig. 6(b)). No cross-enhancement was found with two other dicarboximide fungicides, procymidone or myclozolin.

3.2.6 Pretreatment with 3,5-dichloroaniline

In vinclozolin-pretreated, iprodione-pretreated or untreated soils, 40% of a 3,5-DCA application (25 $\mu g\ g^{-1}$ soil) disappeared within 24 h; thereafter the two fungicide-pretreated soils continued to degrade the remaining 3,5-DCA rapidly (90% of the original application disappeared in four to five days) but subsequent loss of 3,5-DCA in the untreated soil was very much slower; 75% loss required 14 days. The untreated soil containing the respiratory inhibitor sodium azide was, in contrast, almost inactive from zero time and showed only 20% loss of the original 3,5-DCA content after 14 days (Fig. 7). Pretreatment of soil with 3,5-DCA did not stimulate the rates of either iprodione or vinclozolin degradation compared with those in untreated soil (data not shown).

Though the fungicides are not used in combination agriculturally, their rates of degradation by ipriodione- and vinclozolin-pretreated and by untreated soils were examined when both fungicides were mixed together, or with 3,5-DCA. There were only marginal differences between the rates of ipriodione and vinclozolin degradation, or 3,5-DCA disappearance, when each was added in combination rather than individually to pretreated soils, and no differences at all when they were added in combination or individually to untreated soil.

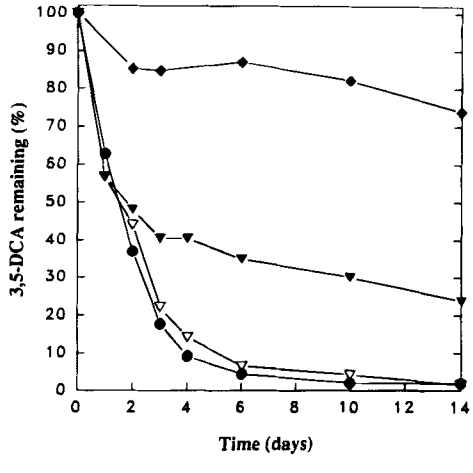


Fig. 7. Disappearance of 3,5-DCA in (▼) soil not previously exposed to any fungicide; (▽) soil previously exposed to vinclozolin; (●) soil previously exposed to iprodione and (◆) untreated soil which had been mixed with sodium azide to a final concentration of 2 mg g^{-1} dry weight of soil.

TABLE 1

The Effect of Antibiotics on the Degradation Constants ($-k_D$) for Iprodione and Vinclozolin in Pretreated and Untreated Close House Soil

Soil treatment	*Antibiotic added*[a]	$-k_D$ (day^{-1})	
1. Untreated, receiving	None	0·014[b]	0·018[c]
iprodione[b] or	Chloramphenicol	0·013[b]	0·062[c]
vinclozolin[c]	Rifampicin	0·030[b]	0·029[c]
2. Iprodione-pretreated,	None	1·616	
receiving iprodione	Chloramphenicol	0·078	
	Cycloheximide	1·575	
	Kanamycin	1·500	
	Nystatin	1·536	
	Rifampicin	0·027	
	Steptomycin	1·432	
3. Vinclozolin-pretreated	None	1·626	
receiving vinclozolin	Chloramphenicol	0·032	
	Kanamycin	0·988	
	Nystatin	1·621	
	Rifampicin	0·026	
	Streptomycin	1·023	

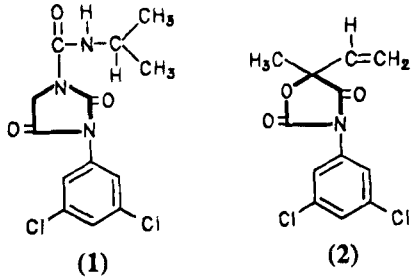
[a] Antibiotics were added at 3 mg g^{-1} soil together with glucose at 5 mg g^{-1} soil and the appropriate fungicides at 25 $\mu g\ g^{-1}$ soil.
[b,c] Results corresponding to treatment as indicated.

3.2.7 The effects of metabolic poisons and antibiotics
The respiratory poison sodium azide, when added to pretreated soil at 2 mg g^{-1} soil, inhibited fungicide degradation within 36 h. The addition of mercuric chloride to soil showing enhanced degradation also halted the degradation of the fungicides within 12 h of its application and maintained this inhibitory effect long after all the fungicide had disappeared in controls with no biocide addition.

In fungicide-pretreated Close House soil, supplemented with yeast extract or glucose, both chloramphenicol and rifampicin reduced the rates of iprodione and vinclozolin degradation to those observed in untreated soil without antibiotic (Table 1). Kanamycin, streptomycin, nystatin or cycloheximide up to 5 mg g^{-1} soil had little effect on iprodione or vinclozolin degradation.

4 DISCUSSION

The microbial nature of the degradation of the dicarboximide fungicides **1** and **2** (Fig. 8) was suggested by the characteristic lag in degradation evident on first application of the fungicides and its elimination with a second or successive treatment (Figs 1 and 2(a), (b)); by the effect of microwaves on those soils exhibiting rapid degradation (Fig. 2) and the eventual reestablishment of degradation in microwaved soil which had not been kept sterile (Section 3.2.1.); by the reversible effects of low moisture levels (Section 3.2.2.) and by the inhibition of fungicide degradation caused by mercuric chloride and sodium azide (Section 3.2.7.). In contrast, the degradation of vinclozolin and iprodione in pretreated soils proved to be difficult to inhibit with several antibacterial antibiotics. The anti-fungal agents cycloheximide and nystatin also had only a minor detrimental effect on fungicide degradation in Close House soil, but the broad-spectrum antibiotic capabilities of chloramphenicol (which inhibits (enzyme) protein synthesis) and rifampicin (which inhibits mRNA transcription), active against both Gram-positive and Gram-negative organisms, were able to attenuate fungicide degradation in the pretreated soils (Table 1). The results suggest a bacterial, rather than a fungal, basis for dicarboximide biodegradation, confirmation of which



(1) (2)

Fig. 8. The structures of (1) iprodione and (2) vinclozolin emphasising their respective hydantoin and oxazolidine moieties.

was achieved by eventual isolation of *Pseudomonas putida* St-1 competent in vinclozolin degradation.[23]

The cultivated soil used in this study, with its history of a total absence of agrochemical input for over 35 years, provided a rare opportunity to investigate the microbial response to the addition of two novel dicarboximide fungicides to that soil, in which organisms specialised for their breakdown could be expected to occur in negligible numbers. In this soil, nevertheless, rapid loss of the initial fungicide treatment, applied at the manufacturer's recommended dose, commenced in about six to eight days and was almost complete at 30 days under field conditions (Fig. 1) and 12–15 days under laboratory conditions at 23°C (Fig. 2(a)). Once-pretreated soil showed no lag in, and accelerated rates of, degradation for a second and subsequent treatment; it thus fulfils the criteria for enhanced degradation. If the principal (or only) agent of vinclozolin degradation in the microflora of this pretreated soil is *P. putida* St-1, [23] then approx. 10^7 cells g^{-1} soil are required to account for the observed rates of fungicide degradation ($-k_D \geq 0{\cdot}3$ day^{-1}) in both soil and cultures. It would require an initial population in the untreated Close House soil of at least $3{\cdot}8 \times 10^5$ cells of *P. putida* g^{-1} soil to attain, with a doubling time of 51 h, a final population of 10^7 cells g^{-1} soil in 8–10 days at the expense of a vinclozolin supplement of 25 μg g^{-1} soil. While the degradation results are thus commensurate with the acute problems which have been encountered in the control by the dicarboximide fungicides of *Sclerotium cepivorum*-mediated white rot of salad onions[4,25] and with reports of enhanced degradation of these fungicides in several other British[10,11,26] and New Zealand[13] soils, the deduced population figure is considerably at variance with the negligible population expected in the Close House soil with its long agrochemical-free history.

In cultures, the heterocyclic ring moieties of these dicarboximide fungicides provide the site of initial microbial attack and their carbon and nitrogen content supports growth of the competent bacteria, because the aromatic moieties of the fungicides accumulate quantitatively as 3,5-dichloroaniline.[23,24] It is evident from the above deduction, that a few specialised cells of this bacterium, inadvertently present in the original agrochemical-free, untreated Close House soil, could not attain the microbial numbers (10^7 organisms g^{-1} soil) necessary to degrade the fungicides at the observed rates in 8–10 days in soil (Section 3.2.1; Table 1)[16–19] or in culture.[23] This time interval could account for only four or five cell doublings. Yet, if a much larger number of soil bacteria (such as the $3{\cdot}8 \times 10^5$ cells g^{-1} soil, calculated above) is responsible for fungicide degradation by means of existing (inducible) enzymes of relaxed specificity,[27–31] rather than by evolving novel catabolic functions *de novo*,[32–34] then the lack of cross-enhancement (Section 3.2.5.) by other dicarboxi-

mides (procymidone, myclozolin, metomeclan and chlozolinate) of very similar structure,[10,11,13,26] and the failure to degrade those same analogues by *P. putida* St-1,[23] remains puzzling. Specificity of the amidohydrolases[29–31] involved in heterocycle catabolism must clearly extend to a recognition of the different substituents on the heterocyclic rings of the dicarboximides, otherwise vinclozolin and myclozolin (CH_2=CH— and CH_3OCH_2—, respectively, at C-5 of the oxazolidine ring) would both be attacked by *P. putida* St-1 and would show cross-enhancement of each other's degradation in soil;[11] neither of these features occurred.

The ease with which enhanced degradation could be established in the untreated Close House soil was emphasised by the requirement for as little as 0·1% (w/w) of active fungicide-degrading soil, mixed with similar untreated soil, to cause a measurable increase (significant at $P < 0{\cdot}01$) in the rate of fungicide degradation by the mixture compared to that in untreated soil alone (Fig. 3). Walker *et al.*[10] found that a similar stimulation in a slowly degrading acid soil needed 10%(w/w) of a pretreated component, whereas in our more typical soil no further increase in fungicide degradation could be effected by increasing the proportion of the pretreated component above 5% (w/w). From the microbial counts (10^7 g^{-1} of soil) of the vinclozolin-degrading bacterium required to effect maximum degradation in untreated soil,[23] a 0·1% (w/w) soil amendment should be equivalent, at most, to an inoculum of only 10^4 degrading bacteria g^{-1} soil and a 5% (w/w) amendment to an inoculum of 5×10^5 cells g^{-1} soil. With a 51-h generation time,[23] the latter inoculum would take approx. 220 h (9·25 days) to reach 10^7 organisms g^{-1} soil if it grew only at the expense of the fungicide; test applications actually disappeared in 10–12 days, in good agreement with the effect of a 5% (w/w) pretreated soil amendment to untreated soil (Fig. 3).

Similarly, the application of very low doses of iprodione or vinclozolin (0·5 μg AI g^{-1} soil), 40–50-fold lower than the recommended rate of field application (1·5 kg AI ha^{-1}, equivalent to 25 μg AI g^{-1} soil) and some 10-fold less than used by others,[4,10,11,26] was still sufficient to stimulate enhanced degradation of subsequent test applications of the fungicide (Fig. 5) in the Close House soil. Although this amount of fungicide is supplying no more than 0·13 μg of utilisable carbon g^{-1} soil (from the heterocyclic ring of the fungicides), it could still support a basic population of $1{\cdot}7 \times 10^5$ fungicide-degrading organisms g^{-1} soil (see Cain & Mitchell[23] for calculations), a number which would need 300 h (12·5 days) to attain the 10^7 organisms g^{-1} soil at which degradation rates of test applications of the fungicides are maximal. This value is close to the experimentally determined time (13·3 days) needed for disappearance of the test application of fungicide following pretreatment with application rates as low as 0·5 μg g^{-1} soil ($-k_D$ 0·15 day^{-1}) (Fig. 5). At the other extreme, an application rate of 700 μg g^{-1} soil is approximately 30-fold higher than recommended field application rates, yet over 90% of an application at this rate was also transformed in pretreated soil in six days for iprodione and nine days for vinclozolin, with little residual 3,5-DCA extractable after nine days, implicating eventual degradation of the aromatic moiety of these fungicides in Close House soil.

Although pretreatment with, or the presence of, metabolites has sometimes been held to stimulate rapid degradation of their parent compound,[35,36] in the experiments reported here, a soil pretreatment with 3,5-DCA had no stimulatory effect on subsequent rates of fungicide degradation nor was there a demonstrable problem with toxicity by 3,5-DCA in the soil experiments (Fig. 7), although dichloroanilines are known to be growth inhibitory at low concentrations to a broad range of micro-organisms.[37] The accumulation of 3,5-DCA was almost quantitative in a vinclozolin-degrading pure culture of *P. putida* St-1[23] and in iprodione-degrading mixed cultures of bacteria,[24] but its concentration eventually fell with time in biologically active soils (Figs 1 and 7). From an application in one experiment (Section 3.2.4.) of 700 μg iprodione g^{-1} of soil, the highest level of 3,5-DCA recorded was 240 μg g^{-1} of soil but this fell to 14 μg g^{-1} of soil after a further five days. While some 3,5-DCA probably volatilises or complexes with the humic matter in soil, as happens with the 3,4-dichloroaniline metabolite of the herbicides propanil, diuron and linuron,[37,38] the biological contribution to 3,5-DCA turnover in Close House soil remained predominant, because soil treated with biocides showed greatly reduced disappearance of 3,5-DCA (Fig. 7).

Examples of cross-enhancement of degradation within several groups of soil pesticides have been widely reported.[1,2] Walker[11] observed degradation of iprodione in vinclozolin-pretreated soil but found no significant vinclozolin degradation in iprodione-pretreated soil, whereas our study found that in soil under optimised laboratory conditions, iprodione was transformed in vinclozolin-pretreated soil and vinclozolin in iprodione-pretreated soil at rates considerably faster than either in untreated soil (Fig. 6). Nevertheless, the stimulation of iprodione removal in vinclozolin-pretreated soil was considerably greater (90% disappearance in four days) than that of vinclozolin in iprodione-pretreated soil (90% disappearance in eight days), a feature which may be related to the fungicide structures. The imidazolidine dione (hydantoin) moiety (Fig. 8) of iprodione, for instance, occurs widely in natural products (e.g. allantoin, uric acid) so enzymes capable of its hydrolysis have probably evolved already in soil micro-organisms. The oxazolidine dione moiety (Fig. 8) of vinclozolin, in contrast, is much rarer among natural products, and the distribution of organisms with

the enzymic capacity to degrade this structure may be less abundant. Iprodione degraders require only amidohydrolases[29,30] to rupture a hydantoin ring; these enzymes do not hydrolyse the intact oxazolidine ring of vinclozolin, though they would act on the (known) intermediate[39] from its lactonic hydrolysis, from which an arylamidohydrolase[10] would generate 3,5-DCA. Amidohydrolases also occur as part of the active soil-enzyme component present in arable soils.[40]

Invoking enhanced fungicide degradation in a non-degrading soil by the addition of 0·1–5% by weight of a degrading soil (Fig. 3) demonstrates the ease of transfer and suggests that its agent(s) proliferate(s). Many hypotheses have been proposed to explain microbial dissemination in the environment.[41–45] In cultivated soils, agricultural workers, machinery, animals and the weather must contribute to the macro-dispersal of soil micro-organisms but transfer between bacteria of xenobiotic-degrading abilities, encoded by genes on transmissible bacterial plasmids, has been suggested as a means by which novel catabolic functions can spread among prokaryotes in the environment.[46–52] This mechanism has already been implicated in the soil biodegradation of some carbamate and organophosphorus insecticides;[49] it may be a factor in the degradation of the dicarboximide fungicides.

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